

Exaggerated aggression and decreased anxiety in mice deficient in brain serotonin

V Mosienko^{1,2}, B Bert³, D Beis^{1,2}, S Matthes^{1,2}, H Fink³, M Bader¹ and N Alenina¹

Serotonin is a major neurotransmitter in the central nervous system (CNS). Dysregulation of serotonin transmission in the CNS is reported to be related to different psychiatric disorders in humans including depression, impulsive aggression and anxiety disorders. The most frequently prescribed antidepressants and anxiolytics target the serotonergic system. However, these drugs are not effective in 20–30% of cases. The causes of this failure as well as the molecular mechanisms involved in the origin of psychological disorders are poorly understood. Biosynthesis of serotonin in the CNS is initiated by tryptophan hydroxylase 2 (TPH2). In this study, we used *Tph2*-deficient (*Tph2*^{−/−}) mice to evaluate the impact of serotonin depletion in the brain on mouse behavior. *Tph2*^{−/−} mice exhibited increased depression-like behavior in the forced swim test but not in the tail suspension test. In addition, they showed decreased anxiety-like behavior in three different paradigms: elevated plus maze, marble burying and novelty-suppressed feeding tests. These phenotypes were accompanied by strong aggressiveness observed in the resident–intruder paradigm. Despite carrying only one copy of the gene, heterozygous *Tph2*^{+/−} mice showed only 10% reduction in brain serotonin, which was not sufficient to modulate behavior in the tested paradigms. Our findings provide unequivocal evidence on the pivotal role of central serotonin in anxiety and aggression.

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Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is a monoamine that has a dual role, working both as an autacoid in the periphery and as a neurotransmitter in the brain. Synthesis of 5-HT starts with the conversion of the essential amino acid tryptophan (Trp) to 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase (TPH). In the second step, 5-HTP is decarboxylated to 5-HT by the aromatic amino acid decarboxylase. The discovery of a second *Tph* gene unraveled the existence of two independent 5-HT systems in vertebrates — peripheral and central — controlled by TPH1 and TPH2, respectively.^{1,2} Although TPH1 is mainly expressed in the gut and is responsible for the synthesis of peripheral serotonin, TPH2 is expressed in the neurons of the raphe nuclei in the brainstem^{2–4} and in myenteric neurons in the gut,⁵ but not in other peripheral organs such as the lung, heart, kidney or liver.^{6,7} Mice lacking TPH2 (*Tph2*^{−/−}, *Tph2*-deficient mice) were recently generated by our group⁸ and others.^{3,9–12} *Tph2*^{−/−} mice exhibit only minute amounts of brain serotonin, but normal formation and differentiation of serotonergic neurons.^{3,8} The level of peripheral serotonin was unchanged in these mice, although it did not restore the brain 5-HT levels as serotonin cannot cross the blood brain barrier. *Tph2*^{−/−} mice display normal size and no obvious abnormalities at birth, but exhibit growth retardation during early postnatal life. However, *Tph2*^{−/−} mice catch up the body weight and at the age of 3 months are not any more

distinguishable from wild-type mice. Moreover, these mice exhibit altered thermoregulation and respiratory control, and impaired maternal care.⁸

It has been postulated that reduction in brain serotonin leads to increased depressive and aggressive behaviors. In humans different polymorphisms of genes involved in the central serotonin synthesis and transmission are associated with various psychological abnormalities such as depression,^{13–17} anxiety disorders¹⁸ and aggression.^{19,20} Moreover, differences in the level of serotonin or its metabolite, 5-hydroxyindoleacetic acid (5-HIAA), in the cerebrospinal fluid have been found in patients with depression^{21–23} and destructive behaviors, such as aggression, violence and impulsivity.^{22,24–26} Further elucidation of mechanisms by which central serotonin is involved in depression and anxiety disorders is required for the improvement of existing medical treatment.

In this study, we evaluated the rate of serotonin synthesis in the central nervous system (CNS) of *Tph2*^{−/−} and *Tph2*-heterozygous (*Tph2*^{+/−}) mice, and investigated the consequence of complete and partial central serotonin depletion on anxiety, aggression and depression-like behavior.

Materials and methods

Animals. All animal procedures were in accordance with the ethical principles and guidelines for care and use of laboratory animals adopted by German local authorities

¹Department of Molecular Biology of Peptide Hormones, Max-Delbrueck-Center for Molecular Medicine, Berlin, Germany; ²Faculty of Mathematics and Natural Sciences, Department of Biology, Humboldt-Universität Berlin, Berlin, Germany and ³Institute of Pharmacology and Toxicology, School of Veterinary Medicine, Freie Universität Berlin, Berlin, Germany

Correspondence: Dr N Alenina, Department of Molecular Biology of Peptide Hormones, Max-Delbrueck-Center for Molecular Medicine, Robert-Rössle Street 10, 13125 Berlin, Germany.

E-mail: alenina@mdc-berlin.de

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corresponding to the standards prescribed by the American Physiological Society.

Mice were maintained in individually ventilated cages, 34 × 19 × 13 cm (Tecniplast Deutschland, Hohenpeissenberg, Germany) under a standard light/dark cycle from 7 am to 7 pm, with free access to standard chow (0.25% sodium, SSNIFF Spezialitäten, Soest, Germany) and drinking water *ad libitum*.

To obtain *Tph2* gene-deleted mice on a pure genetic background, heterozygous *Tph2*-deficient animals on C57Bl/6 background (6th generation)⁸ were bred for further four generations to C57Bl/6 mice (Charles River, Sulzfeld, Germany). All experiments have been performed in adult (18–22 weeks old) F10 C57Bl/6 *Tph2*^{-/-}, *Tph2*^{+/-} and wild-type (*Tph2*^{+/+}) male mice. To generate animals of the three above-mentioned genotypes *Tph2*^{+/-} females were bred with *Tph2*^{-/-} or *Tph2*^{+/+} male mice. Genotyping was performed using PCR with primer TPH34 (5'-AGC TGA GGC AGA CAG AAA GG-3'), TPH54 (5'-CCA AAG AGC TAC TCG ACC TAC G-3') and Neo3 (5'-CTG CGC TGA CAG CCG GAA CAC-3'). Mice were single housed starting at 10–12 weeks of age as *Tph2*^{-/-} males are highly aggressive and cannot be kept in groups. In order to avoid differences due to single-housing condition *Tph2*^{+/-} and *Tph2*^{+/+} were single housed as well at least 4 weeks before experiments.

Group-housed (five mice per cage) 23–25 g male mice on FVB/N background were used as intruders in the resident–intruder paradigm.

Behavioral assessment. Behavioral testing was performed during the light cycle between 1 and 5 pm, with exception of tail suspension test (TST), which was performed during the dark cycle. In all experiments mice were habituated at least for one week to the experimental room. During this time handling of animals was done by the same experimenter who performed the tests. One batch of animals was used for the marble burying test (MBT) and was afterwards tested in the elevated plus maze (EPM) test with a 1 week interval between the two experiments. Another batch of animals was tested first in the novelty-suppressed feeding test (NSF), and 1 week later in the resident–intruder test (RI). Mice tested first in the open field were then examined in the forced swim test (FST). For the TST independent cohorts of naïve animals was used. Before, TST mice were kept 9 weeks under reversed dark–light cycle, with the light off at 10 am. Mouse behavior was video recorded (Panasonic camera HDC TM700, Hamburg, Germany) for subsequent offline analyses by the experimenter. For analysis of EPM and OF data Biobserve software (Viewer² version 2.2.0.91, BIOBSERVE GmbH, Bonn, Germany) was used. In MBT, activity was measured by InfraMot (TSE systems, Bad Homburg Germany). In all experiments, the observer was blinded to the genotype.

Open field. A large arena (50 × 50 cm) under low illumination (30 Lux) was used as an OF to measure locomotor activity. Each mouse was placed into the arena facing the middle of the wall and its activity was measured during 10 min. The total distance traveled, time spent in the center and near the walls were calculated.

Elevated plus maze. The EPM test is based on the inborn aversion of rodents to open, bright illuminated spaces.²⁷ The maze consisted of two open arms (30 × 5 cm) and two closed arms (30 × 5 cm) that were enclosed by a sidewall on all edges (height 15 cm). Mice were placed in the center of the maze (central platform) facing the closed arm. Total arm entries, percent of entries into the open arm ((open-arm entries/total arm entries) × 100) and time spent in open arms ((open arms/total session duration) × 100) were quantified during 10 min test. Arm entry was only defined when an animal (the mouse mass center) was at least 3 cm on an arm to differentiate entries from stretched attend postures into the arms.

Marble-burying test. Marble burying is a common test for validating anxiolytic effect of drugs.²⁸ The test was conducted in a new cage (equally sized and illuminated as the home cage) with evenly spaced 15 clear glass marbles (20 mm diameter) in 5 cm of sawdust. During the test mice had access to food and water, and the test cage was covered with a metal grid. After 30 min the test was terminated by removing the mouse and the number of buried marbles was counted. A marble was scored as buried if more than two-thirds of it was covered with sawdust. During the test locomotor activity was evaluated by InfraMot (TSE systems).

Novelty-suppressed feeding. This test is based on a provoked conflict between the fear of mice to enter bright illuminated spaces and food seeking induced by hunger.^{29,30} Animals were food deprived 23 h before testing. On the test day after placing mice into a novel home cage for 30 min, they were introduced into a new brightly illuminated test environment (cage 42 × 25 × 18 cm) where a single food pellet was centrally placed. After the first feeding event animals were returned to their home cages where they were allowed to eat pre-weighed food over a period of 5 min. Latency to the first eating episode (time between mouse introduction to arena with food pellet in the middle and the first feeding event) was used as an index of induced anxiety-like behavior. The amount of food consumed in the home cage provided a measure of appetitive drive.

Forced swim test. The FST, as originally described by Porsolt,³¹ assesses the tendency to give up attempting to escape from an unpleasant environment, whereby fewer attempts are interpreted as behavioral despair. The apparatus was a plastic beaker (17.5 cm diameter, 24 cm high), filled with water (24–26 °C) to a height of 18 cm. The time mice spent floating on the water (immobility time, sec) during 6 min as well as latency (sec) to the first immobility episode were manually observed. A mouse was judged to be immobile when it ceased struggling and remained floating motionless in water, making only those movements necessary to keep its head above water. Swimming was defined as vigorous movements with forepaws breaking the surface of the water.

Tail suspension test. The TST is another learned helplessness paradigm where animals cannot escape from an unpleasant situation. A reduction in struggling behavior

(latency to the first immobile episode or increased total immobility) is interpreted as a reduction in intrinsic motivation to escape the situation. Mice were suspended by the tail using an adhesive tape to a platform. The latency to the first immobility episode and the duration of immobility over a 6 min period were continually manually measured. An animal was rated as immobile when there was no movement of the head, extremities or the torso.

Resident–intruder test. The RI test is based on the territory defensive behavior against unfamiliar intruding conspecifics.³² Each single-housed resident male was confronted in its home cage by a group-housed (five mice per cage) intruder male FVB/N mouse for 10 min. Each intruder mouse was used only once to avoid submissive/dominance effects after first interaction. Behavioral interactions during each confrontation were recorded and subsequently scored by an observer. Latency to the first attack, total amount of attacks and cumulative duration of attacks were analyzed.

Neurochemical assessments. To prepare brains for high-performance liquid chromatography (HPLC) analysis, animals were anesthetized by intraperitoneal ketamine (100 mg kg⁻¹) and xylazine (10 mg kg⁻¹) injection. Animals were transcardially perfused with phosphate-buffered saline containing 300 U ml⁻¹ heparin (Braun, Melsungen, Germany) to remove the blood, containing peripheral 5-HT. Brains were removed, weighed and snap-frozen on dry ice. For the determination of serotonin and its metabolites, frozen tissues were homogenized in lysis buffer containing 10 μM ascorbic acid and 1.8% perchloric acid, centrifuged for 30 min at 20 000 g, 4 °C, and the supernatant was used for HPLC measurement. Tissue levels of 5-HTP, 5-HT and its metabolite 5-HIAA were analyzed using high sensitive HPLC with fluorometric detection (Shimadzu, Tokyo, Japan).³³ Sample separation takes place at 20 °C on a C18 reversed-phase column (OTU LipoMareC18, AppliChrom Application & Chromatography, Oranienburg, Germany) using a 10 mM potassium phosphate buffer, pH 5.0, containing 5% methanol with a flow rate of 2 ml min⁻¹. Fluorescence of 5-HTP and 5-HT is excited at 295 nm and measured at 345 nm. For the evaluation of serotonin synthesis *in vivo* animals were injected intraperitoneally with 100 mg kg⁻¹ of aromatic amino acid decarboxylase inhibitor 3-hydroxybenzylhydrazine dihydrochloride (NSD-1015, CatNr. 54880, Sigma-Aldrich, Munich, Germany) 1 h before brain dissection. Amounts of 5-HT, 5-HTP and 5-HIAA were normalized to the wet tissue weight for statistical analysis. Calculation of substance levels was based on external standard values.

Real-time-PCR analysis. For real-time-PCR (RT-PCR) analysis animals were first decapitated, and brains were promptly removed and snap-frozen on dry ice. RNA from the whole brain was extracted with Trizol reagent (15596-018 Invitrogen, Darmstadt, Germany), and residual genomic DNA was removed by DNase I treatment (DNA amplification grade, Sigma-Aldrich). RNA was reverse transcribed using random hexamers and modified Moloney murine leukemia virus reverse transcriptase (Superscript II, Invitrogen) according to the manufacturer's protocol. RT-PCR was run

in a technical triplicate using SYBR green reagent (Qiagen, Hilden, Germany) in a 384-well plate format (fast RT-PCR system 7900HT, Applied Biosystems, Darmstadt, Germany). The expression of the *Tph2* gene was quantified using RT² quantitative PCR primer assay (PPM27894A-200 SABioscience, Hilden, Germany). *Tph2* expression was normalized to TATA-binding protein (TBP) mRNA expression (primers: forward 5'-CCC TAT CAC TCC TGC CAC ACC-3', reverse 5'-CGA AGT GCA ATG GTCTTT AGG TC-3'). The method of Livak and Schmittgen³⁴ was applied to compare gene expression levels between groups, using the equation $2^{-\Delta\Delta CT}$.

Statistics. Results are expressed as mean ± s.e.m. Statistical analysis was performed by unpaired Student's *t*-test and by one way ANOVA with Bonferroni's correction as a post-hoc test for multiple comparisons (PRISM, GraphPad, San Diego, CA, USA). *P* < 0.05 was considered to be significant.

Results

***Tph2* expression and serotonin levels in *Tph2*^{-/-} mice.** We first evaluated the amount of *Tph2* transcripts in the brains of *Tph2*^{+/-} and *Tph2*^{+/+} mice, containing one and two copies of the *Tph2* gene, respectively. RT-PCR showed a 50% reduction in *Tph2* gene expression in the whole brain of *Tph2*^{+/-} mice in comparison with *Tph2*^{+/+} mice (Figure 1a). We next measured the amount of serotonin and its degradation product, 5-HIAA, in the whole brain of *Tph2*^{-/-}, *Tph2*^{+/-} and *Tph2*^{+/+} mice by HPLC. *Tph2*^{-/-} mice contained <2% of *Tph2*^{+/+} 5-HT level and no detectable 5-HIAA in the brain (Figure 1b, Table 1), confirming previous results.⁸ However, only around 10% reduction in brain serotonin levels was observed in *Tph2*^{+/-} in comparison with *Tph2*^{+/+} mice, whereas the level of 5-HT degradation product, 5-HIAA, was reduced nearly by half in *Tph2*^{+/-} (Figure 1b, Table 1). We further evaluated the 5-HT synthesis rate in *Tph2*^{-/-}, *Tph2*^{+/-} and *Tph2*^{+/+} mice by blocking conversion of the 5-HTP to 5-HT by the aromatic

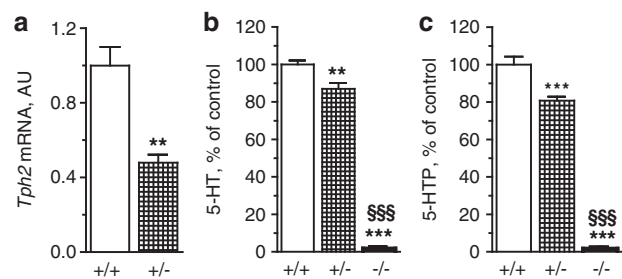


Figure 1 *Tph2* expression and serotonin synthesis in *Tph2*^{-/-} mice. **(a)** RT-PCR analysis of *Tph2* expression in the brain. AU, arbitrary units. ***P* < 0.01, Student's *t*-test. **(b)** 5-HT level in the whole brain (HPLC measurement). *Tph2*^{+/+} 5-HT level is taken as 100%. **(c)** 5-HTP level in the whole brain 1 h after NSD administration (100 mg kg⁻¹, intraperitoneally) (HPLC measurement). *Tph2*^{+/+} 5-HTP level is taken as 100%. Data are shown as means ± s.e.m., *n* = 6. ****P* < 0.001, ***P* < 0.01 vs *Tph2*^{+/+}, §§§*P* < 0.001 vs *Tph2*^{+/-}, one-way ANOVA with Bonferroni correction. ANOVA, analysis of variance; HPLC, high-performance liquid chromatography; 5-HT, 5-hydroxytryptamine; 5-HTP, 5-hydroxytryptophan.

Table 1 Serotonin, 5-HIAA and 5-HTP in the brains of *Tph2*-deficient mice

	5-HT (pg mg ⁻¹)	5-HIAA (pg mg ⁻¹)	5-HTP (pg mg ⁻¹)	5-HTP/NSD (pg mg ⁻¹)
<i>Tph2</i> ^{+/+}	753.0 ± 16.3	366.3 ± 34.9	3.7 ± 0.3	329.1 ± 14.0
<i>Tph2</i> ^{+/-}	655.6 ± 24.1**	217.0 ± 11.7*	2.0 ± 0.2*	266.7 ± 7.1*
<i>Tph2</i> ^{-/-}	13.6 ± 1.0***	0.0 ± 0.0***	0.0 ± 0.0***	5.7 ± 0.3***

Abbreviations: 5-HT, 5-hydroxytryptamine; 5-HTP, 5-hydroxytryptophan; 5-HIAA, 5-hydroxyindoleacetic acid; NSD, 3-hydroxybenzylhydrazine dihydrochloride. Serotonin (5-HT), 5-HTP and 5-HIAA levels were measured by HPLC in whole brain lysates. Serotonin synthesis rate was evaluated by the accumulation of 5-HTP in the brain during 1 h after administration of the AADC inhibitor, NSD (5-HTP/NSD column). Values are normalized to mg of wet brain tissue. Data are presented as means ± s.e.m., *n* = 6. **P* < 0.001; ***P* < 0.01 vs *Tph2*^{+/+}; ****P* < 0.001 vs *Tph2*^{-/-} (one-way ANOVA with Bonferroni's correction).

amino acid decarboxylase inhibitor NSD. An around 20% decrease in accumulation of 5-HTP was observed in *Tph2*^{+/-} in comparison with *Tph2*^{+/+} mice (Figure 1c, Table 1). As expected, *Tph2*^{-/-} mice accumulated <2% of 5-HTP compared with *Tph2*^{+/+} (Figure 1c, Table 1).

Anxiety-like behavior in *Tph2*^{-/-} mice. We first evaluated overall activity of *Tph2*^{-/-} mice in the OF under low illumination conditions. *Tph2*^{-/-} mice did not show any difference in locomotor activity in comparison with *Tph2*^{+/-} and *Tph2*^{+/+} mice (Figure 2a).

In EPM, *Tph2*^{-/-} mice spent significantly more time in the open arms than *Tph2*^{+/+} and *Tph2*^{+/-} (*P* = 0.0161 and *P* = 0.0133, respectively) (Figure 2c). *Tph2*^{-/-} mice also exhibited twice the amount of open-arm entries compared with *Tph2*^{+/+} (*P* = 0.0026 vs *Tph2*^{+/+}, *P* = 0.0054 vs *Tph2*^{+/-}) (Figure 2d). However, total arm entries and total distance traveled were comparable between mice of all three genotypes (Figure 2b). Analysis of locomotion in the EPM over time showed that *Tph2*^{-/-} mice extensively explored the brightest illuminated part of the open arms already during the first 5 min of testing, whereas *Tph2*^{+/+} animals did not enter the distal parts of the open arms during the whole 10 min of the test. *Tph2*^{+/-} mice did not show any significant difference compared with *Tph2*^{+/+} mice neither in the total time spent in open arms nor in the open-arm entries (Figure 2c and d).

The amount of marbles buried by *Tph2*^{-/-} mice in the MBT was significantly lower than that of *Tph2*^{+/+} and *Tph2*^{+/-} animals (*P* = 0.0199 and *P* < 0.0001, respectively) (Figure 2e). Interestingly, the general activity of *Tph2*^{-/-} animals during this test was almost twofold higher than that of *Tph2*^{+/+} (*P* = 0.0046) (Figure 2f). There was no significant difference in the percentage of marbles buried by *Tph2*^{+/-} mice compared with *Tph2*^{+/+} mice (Figure 2e). However, *Tph2*^{+/-} showed an intermediate activity, significantly different from both *Tph2*^{-/-} and *Tph2*^{+/+} mice (*P* = 0.009 and *P* = 0.023, respectively) (Figure 2f).

In the NSF task, *Tph2*^{-/-} mice needed less time to reach and start eating the food pellet in the center of the arena compared with *Tph2*^{+/+} and *Tph2*^{+/-} (*P* = 0.002 and *P* = 0.017, respectively) (Figure 2g). Food consumption, evaluated during 5 min following the test did not differ between the genotypes (Figure 2h). *Tph2*^{+/-} mice did not show a significant difference in the latency to reach and start eating the food in comparison with both, *Tph2*^{+/+} and *Tph2*^{-/-} (Figure 2g and h).

Depression-like behavior in *Tph2*^{-/-} mice. In the FST, *Tph2*^{+/-} mice did not show any significant difference in

comparison with *Tph2*^{+/+} in the total immobility time or the latency to the first immobility episode, whereas *Tph2*^{-/-} mice demonstrated reduced struggling behavior (Figure 3). They spent less time swimming until the first immobility episode (*P* = 0.0001) (Figure 3a) and stayed longer immobile compared with *Tph2*^{+/-} and *Tph2*^{+/+} littermates (*P* = 0.005, in comparison with both genotypes) (Figure 3b). Moreover, *Tph2*^{-/-} mice showed an increase in immobility time during each single 2 min episode compared with *Tph2*^{+/+} mice (Figure 3b). In the TST no significant differences between genotypes could be found neither in the latency to immobility, nor in the struggling time (Figure 3c and d).

Aggressive behavior in *Tph2*^{-/-} mice. In the RI test, *Tph2*^{-/-} mice attacked the intruder almost six times faster than *Tph2*^{+/+} mice (*P* = 0.0002) (Figure 4a). Furthermore, the number of attacks and the cumulative attack duration in the *Tph2*^{-/-} vs *Tph2*^{+/+} group were elevated sevenfold (*P* = 0.0014 and *P* = 0.01, respectively) (Figure 4b and c). A qualitative analysis of attacks revealed a striking difference between *Tph2*^{-/-} and *Tph2*^{+/+} mice: within 5 min of the test all mutant animals displayed aggressive bouts, while only 22% of *Tph2*^{+/+} mice showed such behavior. Though *Tph2*^{+/-} mice tended to show an intermediate state of aggressive behavior between *Tph2*^{-/-} and *Tph2*^{+/+} mice, neither the differences in the first attack latency nor the number of attacks between *Tph2*^{+/-} and *Tph2*^{+/+} mice were significantly different (Figure 4a and b).

Discussion

Although the implication of brain serotonin in animal behavior has been recognized already in the last century, most of the studies were conducted using pharmacological or genetic inhibition of serotonin reuptake and 5-HT receptors. However, the role of serotonin *per se* in these studies was not completely clarified, because no suitable animal model was available yet. In this study, we used mice deficient in brain serotonin synthesis on a pure genetic background to evaluate the consequences of complete absence of this neurotransmitter in the CNS on aggression-, depression- and anxiety-like behavior.

A role of serotonin in the etiology of depressive disorders was suggested more than 50 years ago.³⁵ Later on, formulation of the monoaminergic theory of depression led to the development of antidepressive drugs, which increase the monoaminergic activity.³⁶ Moreover, severely depressed patients treated with Trp or 5-HTP show symptomatic improvement,^{37,38} whereas, giving Trp-free diet to depressed

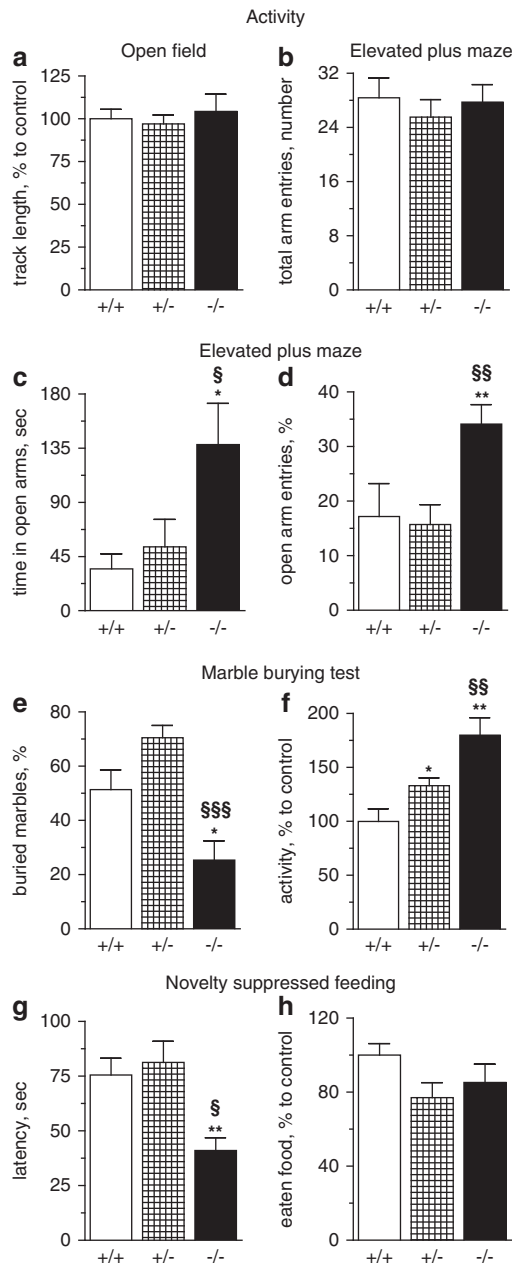


Figure 2 Locomotor activity and anxiety-like behavior in *Tph2*^{-/-} mice. Open field: (a) Total distance traveled. Elevated plus maze: (b) Activity: total number of entries to closed and open arms. (c) Percentage of entries to open arms: entries to open arm/total arm entries. (d) Total time spent in open arms. Marble burying test: (e) Percentage of buried marbles: buried/introduced amount of marbles. (f) Locomotor activity during MBT. Novelty suppressed feeding: (g) Latency to feed. (h) Consumed amount of food during 5 min after reaching the food pellet. (a–f): *Tph2*^{-/-} *n*=10, *Tph2*^{+/-} and *Tph2*^{+/+} *n*=12; (g, h): *Tph2*^{-/-} *n*=11, *Tph2*^{+/-} *n*=13, *Tph2*^{+/+} *n*=10. Data are shown as means ± s.e.m. ***P*<0.01, **P*<0.05 vs *Tph2*^{+/+}; \$\$\$*P*<0.001, §§*P*<0.01, §*P*<0.05 vs *Tph2*^{+/-}, one-way ANOVA with Bonferroni correction. ANOVA, analysis of variance.

individuals elicits a relapse in patients getting treatment with antidepressants.^{39,40}

In our experiments, mice depleted in brain serotonin exhibited a lack of motivation to struggle in the FST that can be interpreted as a depression-like phenotype, supporting

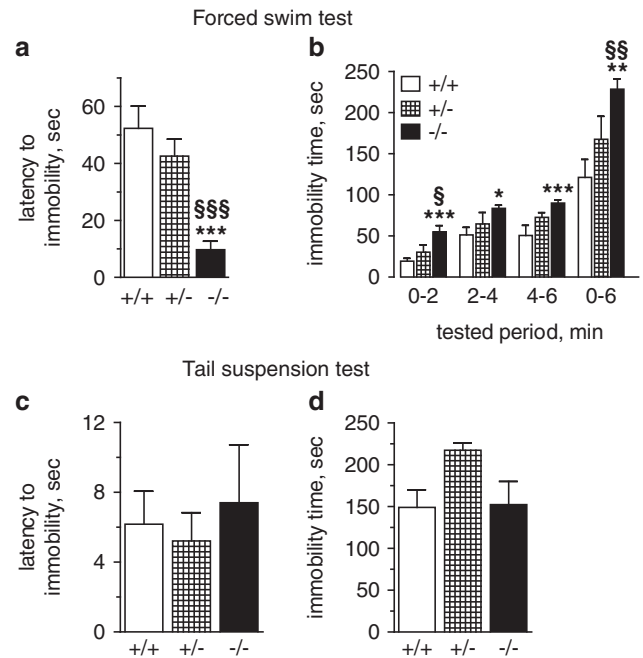


Figure 3 Depression-like behavior in *Tph2*^{-/-} mice. Forced swim test: (a) Latency to the first immobility episode. (b) Immobility time during 2 min intervals and the whole 6 min of tested period. Tail suspension test: (c) Latency to the first immobility episode. (d) Immobility time during 6 min of tested period. Data are shown as means ± s.e.m., (a, b): *n*=10, (c, d): *n*=9. ****P*<0.001, ***P*<0.01, **P*<0.05 vs *Tph2*^{+/+}; \$\$\$*P*<0.001, §§*P*<0.01, §*P*<0.05 vs *Tph2*^{+/-}, one-way ANOVA with Bonferroni correction. ANOVA, analysis of variance.

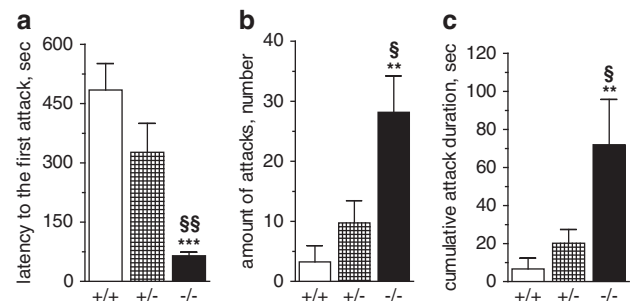


Figure 4 Aggressive behavior in *Tph2*^{-/-} mice. Resident-intruder test: (a) Attack latency: time between the introduction of intruder and the first attack of the resident. (b) Total amount of attacks by the resident. (c) Cumulative attack duration during 10 min tested time. Residents were *Tph2*^{-/-} (*n*=9), *Tph2*^{+/-} (*n*=8), *Tph2*^{+/+} (*n*=7). Data are shown as means ± s.e.m., *n*=8. ****P*<0.001, ***P*<0.01 vs *Tph2*^{+/+}; §*P*<0.05, §§*P*<0.01 vs *Tph2*^{+/-}, one-way ANOVA with Bonferroni correction. ANOVA, analysis of variance.

the monoaminergic theory of depression. There is some discrepancy regarding this phenotype between our results and recent data showing a slight antidepressive effect in *Tph2*^{-/-} mice on the second day of FST.¹⁰ These conflicting findings could be due to several reasons, such as different analysis methods — automated¹⁰ vs manual (our study)⁴¹ — or the 2-day FST protocol,¹⁰ which is commonly used for identifying a depressive state in rats vs the 1-day protocol usually performed for mice.^{42,43} Moreover, the study of depression-like behavior by Savelieva *et al.*¹⁰ was performed

on a mixed genetic background (C57BL/6Jx129S5/S), that may have masked the behavioral effect of *Tph2* gene ablation.

Evaluation of mouse behavior in another widely used paradigm, the TST, did not reveal a depression-like phenotype in *Tph2*^{-/-} mice. This finding is consistent with previous studies showing that depletion of serotonin using p-chlorophenylalanine (PCPA) does not change the outcome of the TST, whereas inhibition of catecholamine synthesis has a prodepressive effect in this test.⁴⁴ Also in the first description of *Tph2*^{-/-} mice¹⁰ no differences in the TST were observed at the first day of experiment. Interestingly, there are reports that show an increased immobility time in the TST in another genetic model of central serotonin depletion — heterozygous VMAT2-knockout mice.⁴⁵ However, in these mice levels of other neurotransmitters are also changed and, therefore, the altered behavior in TST could not be interpreted as only due to the depletion of central serotonin. Surprisingly, when VMAT2 was ablated only in SERT-positive neurons, the behavior in TST was reversed: VMAT2^{SERT-Cre} mice showed a clear antidepressive phenotype in the TST.⁴⁶ However, these animals were on a mixed genetic background that may have veiled the effect of central serotonin ablation.

Although both tests, TST and FST, are widely used for the screening of antidepressants, the validity of these tasks to evaluate symptoms of intrinsic depressive behavior is not so clear.⁴⁷ Moreover, the sensitivity of these two tests to pharmacological drugs is not identical, indicating that different neurochemical pathways may mediate the performance in these tasks.⁴⁸ Additionally, mice being in two different inescapable situations (wet conditions in FST and dry in TST) could use different strategies to struggle. Accordingly, the direction of alterations in depression-like behavior observed in several hyposerotonergic models was not consistent across the studies and even controversial between the two tests (TST and FST) in frames of the same study.^{10,46,49} We observed a clear depression-like phenotype in FST, which was also highly reproducible in our hands—we obtained the same results in two independent experiments (data not shown). However, we could not confirm the depression-like phenotype of *Tph2*^{-/-} mice in the TST. We cannot exclude that this phenotype was masked due to the performance of the test during the dark cycle. Further studies are required to clarify the impact of the dark–light cycle on the depression-like behavior in *Tph2*^{-/-} animals.

Several previous studies failed to detect any drastic alteration in depression-like behavior in models of serotonin depletion after PCPA treatment.^{50–52} The clear depression-like phenotype observed in the FST in *Tph2*^{-/-} mice can be a consequence of a life-long depletion in serotonergic transduction vs short-term effects of PCPA. In this respect, it is interesting to note, that mice prenatally exposed to PCPA show increased depression-related behavior in FST and TST and decreased anxiety.^{53,54} Due to the extreme aggressiveness of *Tph2*^{-/-} mice, animals used in our study could not be kept in groups and were single housed starting 10–12 weeks of age. We can also not exclude that alterations observed in the FST were primed by the prolonged single housing of animals, which may have had more pronounced consequences in *Tph2*^{-/-} animals owing to their higher sensibility to isolation. In addition, hormonal changes resulting from

exaggerated aggressiveness or higher sensitivity to stress, as well as the reduced fat content in *Tph2*^{-/-} animals^{10,12} (our unpublished data) may have had an impact on the outcome of the FST.

As any of the behavior tests used in this study could be influenced by changed activity, we examined whether *Tph2*^{-/-} mice have any alterations in locomotion. Neither activity in the OF (new environment), nor home cage activity measured by telemetry recording⁸ or InfraMot system (TSE Systems GmbH, data not shown) were different between *Tph2*^{-/-} mice and control animals.

Serotonin has been postulated to have a role in aggression.^{55,56} Low cerebrospinal 5-HIAA was correlated with elevated aggression in humans^{26,57,58} and monkeys.⁵⁹ Furthermore, low-Trp diet resulted in increased aggressive behavior in humans,⁶⁰ whereas Trp-enriched diet initiated a reduction of physical aggression in subjects that had a history of elevated aggression.⁶¹ Several genetic variations in serotonergic genes have been linked to impulsive aggression in humans.^{62,63} Moreover, a positive correlation between low serotonin release and increased aggression was confirmed by microdialysis in freely moving animals during the RI test.^{64,65} Inhibition of serotonin synthesis in rats led to increased aggressiveness, whereas enhancement of serotonin transmission suppressed aggressive behavior.⁶⁶ Our study revealed that central serotonin deficiency led to highly increased aggressive behavior in mice. Interestingly, this phenotype was observed not only in males, but also in *Tph2*^{-/-} females.⁸ Thus, our model provides strong evidence for increased aggression as a consequence of complete serotonin deficiency in the CNS being in line with two recently published hyposerotonergic animal models, *TPH2* R439H knockin mice, bearing a single-nucleotide mutation, equivalent to a rare human variant (R441H) identified in depressed patients,⁴⁹ and *Pet-1* deficient animals, which lack most serotonergic neurons.^{62,67} Altogether, these data argue for a direct correlation between the serotonin content in the brain and the level of aggression.

It was recently reported that the absence of brain serotonin leads to increased male–male mounting behavior in a 30 min social interactions task.⁹ This phenotype was not prominent during 10 min of the resident–intruder test performed in our study. Moreover, in several cases we had to interrupt the test due to the extreme aggressiveness of *Tph2*^{-/-} animals. It can not be excluded that defensive behavior of serotonin-deficient animals was misinterpreted in the study of Liu *et al.*⁹

The behavioral evaluation performed in this study showed that *Tph2*^{-/-} mice have decreased levels of aversive behavior in approach-avoidance-conflict tests, correlating with the hypothesis that enhanced serotonergic transmission in the brain facilitates anxiety, whereas a decrease in extracellular 5-HT leads to reduced anxious behavior. This hypothesis, formulated in early 1970s⁶⁸ was further refined using animal models with 5-HT depletion by serotonin synthesis inhibition or lesions of serotonergic neurons.^{69–72} Furthermore, studies in SERT overexpressing and SERT-deficient mice,^{73–75} in 5-HT1a-deficient animals,⁷⁶ as well as in very recently published hyposerotonergic mouse models including *Lmx1b*-, *Pet1*- or *VMAT2*-deficient animals^{46,77,78} correlate with this

hypothesis. Despite being in line with the low-anxiety phenotype, observed in the EPM and NSF tests, the results of the MBT poorly correlate with literature data from other genetic models affecting the serotonergic system.^{10,62,79} We suppose that opposite effects observed in our study originate mostly from the differences in the genetic background (pure C57Bl/6 used by us vs mixed in other studies)—a factor which may strongly affect serotonin-related behavior, as already shown in SERT-knockout mice.⁷⁴ On the other hand, the experimental setup used by us was not identical to the one of other studies: the protocols differ in several aspects, such as amount of marbles, cage parameters and test conditions. Moreover, we cannot exclude that increased locomotion, unexpectedly observed during MBT and not reported in other studies, had an impact on results of this test in our experiments.

There is a vast amount of data about the contribution of molecular variants of *TPH2* to psychiatric disorders in humans.¹⁵ Interestingly, one single-point mutation (R441H) found in a human cohort of late-onset depression was shown to markedly decrease activity of TPH2 and central serotonin level.⁸⁰ A genetic mouse model carrying a single-point mutation (R439H) in *Tph2*, analogous to this human mutation exhibit significantly decreased tissue levels and synthesis rates of 5-HT in the brain, and shows pronounced depression-like behavior in TST, as well as increased aggression.⁴⁹ To check whether reduction in *Tph2* gene copy number may also significantly influence behavior in mice, we evaluated the phenotype of *Tph2*^{+/-} animals. Quantification of *Tph2* mRNA level revealed a decrease in *Tph2* expression by half, suggesting that in wild-type animals both *Tph2* alleles are functional and do not undergo epigenetic modification. Regardless, the 50% decrease in *Tph2* transcriptional activity, only a 10% reduction in 5-HT level was observed. We missed this difference in our previous study,⁸ probably because it was masked by the more heterogeneous background of these animals. Such a slight decrease can be partially explained by a reduced turnover of serotonin by MAO in *Tph2*^{+/-} mice, that is evident from the reduced level of the serotonin degradation product 5-HIAA (Table 1). However surprisingly, evaluation of 5-HT synthesis rate also revealed only a 20% decrease in *Tph2*^{+/-} animals, which probably reflects the limited availability of Trp in the brain.^{81,82} Nevertheless, the 10% decrease in brain 5-HT was not sufficient to alter mouse behavior: *Tph2*^{+/-} were not different from *Tph2*^{+/+} mice in aggression, anxiety or depression-like behavior. Similar effects were recently observed in mice carrying the C1473G mutation in the *Tph2* gene. This mutation resulted in a decreased 5-HT synthesis rate, but hardly changed serotonin content in the brain, and did not affect the behavior in depression and anxiety paradigms.^{83,84} These findings suggest that a lack of one *Tph2* allele alone is not sufficient to modulate aggression and depression-like behavior and therefore is unlikely to be of physiological significance. However, it cannot be excluded that genetic variation in other serotonin-related genes, restriction or alterations in nutrition, medical treatment and epigenetic modifications acquired during lifespan, may unmask a critical role of *TPH2* hypo-expression in the development of pathological symptoms in human.

Taken together, using *Tph2*^{-/-} mice on a pure genetic background, we provide strong evidence that central

serotonin deficiency leads to exaggerated aggression and decreased anxiety and confirm that our animal model is useful to draw unequivocal conclusions about the physiological significance of this neurotransmitter.

Conflict of interest

The authors declare no conflict of interest.

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